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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 February 2002 (07.02.2002)

PCT

(10) International Publication Number
WO 02/09753 A1

(51) International Patent Classification[7]: A61K 39/395, 38/17, 39/42, A61P 31/18

(21) International Application Number: PCT/DK01/00525

(22) International Filing Date: 2 August 2001 (02.08.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PA 2000 01162 2 August 2000 (02.08.2000) DK

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, DZ, EC, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: COMPOSITION AND METHOD FOR THE TREATMENT OR PREVENTION OF HIV INFECTION

(57) Abstract: Therapeutic composition and method for treating and preventing HIV infection, the composition comprising and active substance, which is capable of interacting with a complex of cell-uPAR-uPA-gp120-HIV so as to prevent or reduce the internalisation of HIV into the cell.

WO 02/09753 A1

Composition and method for the treatment or prevention of HIV infection

TECHNICAL FIELD OF THE INVENTION

The present invention relates to a composition for the treatment of HIV infection.

BACKGROUND OF THE INVENTION

The cellular receptor for urokinase (uPAR, CD87) plays multiple functions in cell migration, cell adhesion, pericellular proteolysis and tissue remodeling [Blasi, 1997]. uPAR is expressed by most leukocytes including monocytes, macrophages, neutrophils and platelets. uPAR is an activation antigen in monocytes and T cells [Min, 1992][Nykjaer, 1994] and T-cells from HIV-1 infected individuals express elevated levels of uPAR [Nykjaer, 1994]. HIV-1 infection of leukocytes *in vitro* causes up-regulation of uPAR cell surface expression in a process which appear to be coordinated temporally with the onset of viral replication [Frank, 1996][Speth, 1998]. uPAR may be shed from the cell surface generating a soluble form of the receptor (suPAR) lacking the glycerol-phosphoinositol (GPI) anchor.

Urokinase-type plasminogen activator (uPA) is a protease, which is e.g. involved in converting plasminogen to plasmin.

WO 90/12091 discloses the fact that plasminogen activator inhibitor PAI-1 and uPA form a complex, which binds to uPAR, and that this leads to internalisation of uPA and possibly the whole complex. In general the document teaches that it is possible to inhibit the binding of uPA to uPAR in order to block or reduce the plasminogen activation, and that it is possible in this manner to obtain a therapeutic effect for e.g. cancer.

WO 99/09161 and WO 98/07735 disclose a plasminogen activator inhibitor and the use of the inhibitor for diagnostic and therapeutic applications in connection with a number of diseases, including cancer and HIV infection. The plasminogen activator to be inhibited is e.g. urokinase. The application of the inhibitor for treating HIV infection is explained by the fact that protease inhibitors are effective antiviral

agents. WO 99/09161 and WO 98/0735 do not contain any indication of the mechanism of HIV infection of a cell.

WO 98/42733 discloses peptides comprising an amino acid sequence derived from uPAR, the peptides being useful for the treatment of cancer, autoimmune diseases, hyperinflammatory diseases etc. WO 98/74733 suggests that a cleaved form of soluble uPAR (suPAR) is involved in HIV infectivity.

WO 97/24453 discloses a chimeric polypeptide for internalisation of a virus in a target cell, the polypeptide consisting of a portion of a viral surface polypeptide and a portion of a ligand capable of binding to a cell receptor of the low density lipid (LDL) receptor family. The ligand may e.g. be plasminogen activator inhibitor PAI-1, in which case the mechanism of virus internalisation is stated to be as follows: PAI-1 binds to uPAR-bound uPA to form free complex uPA-PAI-1, which then binds to the LDL receptor, after which internalisation of the complex takes place.

WO 96/13160 discloses a method of inhibiting the infectivity of HIV in a liquid by exposing the liquid to uPA at a selected concentration for a selected period of time sufficient to inactivate HIV in the liquid.

Handley et al. (1) disclose a study of the role of urokinase-type plasminogen activator (uPA) in human immunodeficiency virus type 1 (HIV-1) infection of macrophages. It was shown that uPA, a proteinase which activates plasminogen, binds tightly to a viral envelope protein of HIV-1 named gp120 at its so-called V3 loop, and that uPA can also cleave gp120 within its V3 loop. These reactions can be inhibited with a variety of inhibitors targeted to either side of the interaction between uPA and viral envelope. It was further found that addition of exogenous uPA to a IIIB strain of HIV-1 followed by infection of macrophages with the treated virus resulted in a severalfold increase in subsequent replication. It was speculated that the association of gp120 with uPA may be critical to the fusion of HIV-1 with macrophages, and that the presence of HIV-1 induces the expression of uPA.

Moore et al. address the subject of the HIV-cell fusion reaction. The reference suggests that the fusion of host cell and HIV-1 is mediated by binding of gp120 to

the receptor CD4, after which structural changes that permit an N-terminal hydrophobic peptide of gp41 to penetrate the lipid layer are believed to occur.

SUMMARY OF THE INVENTION

The technical problem addressed by the present invention is to provide a therapeutic composition for preventing and/or treating HIV infection.

This technical problem has been solved with the composition of the present invention, the composition comprising an active substance, which is capable of interacting with the complex of cell-uPAR-uPA-gp120-HIV so as to prevent or reduce the internalisation of HIV.

The present invention is based on the novel discovery that the infection of uPAR expressing cells with HIV is mediated by the forming of a bridge consisting of cell-uPAR-uPA-gp120-HIV to attach HIV to the cell. Following the attachment of HIV to the cell HIV somehow penetrates the cell membrane, the precise mechanism of this penetration not being known. However, it is believed that the formation of the uPAR-uPA-gp120-HIV complex serves to bring the HIV particle in close proximity to a receptor, such as CD4 and CCR5/CXCR4, which mediates the uptake of the HIV particle in the cell.

The invention is further based on the recognition that the infection of cells with HIV may be inhibited by blocking the formation of the bridge consisting of cell-uPAR-uPA-gp120-HIV at any point and in any manner. Secondly, the infection of cells with HIV may be reduced or prevented by compounds, which breaks up one of the bonds in the complex of cell-uPAR-uPA-gp120-HIV. Thirdly, the infection of cells with HIV may be reduced or prevented by compounds, which bind or adhere to the complex of cell-uPAR-uPA-gp120-HIV in such a manner that the internalisation of HIV is affected.

Thus, more specifically the present invention relates to a composition, wherein the active substance is capable of a) interacting with the formation of the complex of cell-uPAR-uPA-gp120-HIV, b) breaking up at least one of the bonds in the complex

of cell-uPAR-uPA-gp120-HIV, and/or c) bonding or adhering to the complex of cell-uPAR-uPA-gp120-HIV so as to prevent or reduce the internalisation of HIV.

The invention further relates to the use of a substance for preparing a therapeutic composition for treating or preventing HIV infection, wherein the substance is capable of interacting with the complex of cell-uPAR-uPA-gp120-HIV so as to prevent or reduce the internalisation of HIV.

The invention further relates to a method of preventing or treating HIV infection comprising administering to a subject a composition comprising a therapeutically effective dose of an active substance, which is capable of interacting with the complex of cell-uPAR-uPA-gp120-HIV so as to prevent or reduce the internalisation of HIV.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 describes the effect of anti-UPAR monoclonal antibody (R3) on HIV protein production after 4 days of culture. Each column shows 8 experiments and the standard deviation is shown as whispers.

Figure 2 describes the effect of monoclonal R5 antibody on HIV protein production. Each column shows 8 experiments and the standard deviation is shown as whispers.

Figure 3 describes the effect of polyclonal rabbit uPAR antibody on HIV protein production. Each column shows 8 experiments and the standard deviation is shown as whispers.

Figure 4 describes the effect of different concentrations of uPA on HIV protein production. U937 cells were pre-incubated with uPA for one hour before addition of HIV-1. Each column shows 8 experiments and the standard deviation is shown as whispers.

DETAILED DESCRIPTION OF THE INVENTION

In the following, some of the preferred active substances for use in the present invention are mentioned and grouped. However, it should be noted that the scope of the invention is not restricted to the specific substances mentioned.

1. Substances interacting with uPAR

Preferably, the active substance of the present invention may be a polyclonal or monoclonal antibody to uPAR. The antibodies may be prepared as described in WO 90/12091.

The active substance of the present invention may be a derivative of uPA capable of binding uPAR and incapable of mediating the infection of HIV into the cell, e.g. 1) uPA having an amino acid sequence, which has been amended by a substitution, a deletion, a truncation and/or an addition, 2) chemically modified uPA and 3) synthetic molecules comprising the amino acid sequence of uPA corresponding to the uPAR binding site of uPA (amino acids 12-32).

Also, the active substance of the invention may be a complex of uPA and PAI-1 or PAI-2. Such complexes will bind to uPAR and cause internalisation of the complexes and presumably also of uPAR hence inactivating uPAR for binding uPA in connection with HIV infection.

Furthermore, the active substance of the invention may be a derivative of pro-uPA capable of binding to uPAR and incapable of being converted to uPA, e.g derivatives modified in the uPA sequence portion cleaved by plasmin. Such substances will bind to uPAR thus blocking the binding of uPA to uPAR.

Further, the active substance of the invention may be a substance, which causes the removal of uPAR from the cell surface by destroying the glycerol-phosphoinositol anchor, such as a phospholipase, e.g. PI-PLC.

Finally, the active substance of the invention may be a substance, which modify the binding affinity of uPAR for uPA, such as phorbol ester PMA and EGF. Such substances will inhibit uPA binding to uPAR, and hence reduce HIV infection.

2. Substances interacting with uPA

The active substance of the invention may be PAI-1 and PAI-2. PAI-1 and PAI-2 are able to bind to both free uPA and uPA bound to uPAR. The binding of PAI-1 and PAI-2 to uPA has been shown to inhibit uPA, and also, it induces the internalisation of receptor-bound uPA making it unavailable to HIV infection.

Also, the active substance may be a substance inducing PAI-1 synthesis, such as hormones, growth factors, cytokines etc., thereby causing uPA degradation and internalisation.

Furthermore, the active substance may be a substance inducing dimerisation of uPAR, such as PAI-1 dimers. It is believed that dimerisation of receptors leads to internalisation and intracellular degradation of uPA.

In a preferred embodiment of the invention, the active substance is a monoclonal or polyclonal antibody to other parts of uPA than the gp120 and uPAR binding sites of uPA, e.g. an antibody to the catalytically active site region of uPA, cf. WO 90/12091.

2a. Substances interacting with the uPAR binding site of uPA

In a preferred embodiment of the invention, the active substance is a monoclonal or polyclonal antibody to the uPAR binding site of uPA.

Also, the active substance may be uPAR molecules and uPAR derivatives capable of binding uPA, e.g. 1) uPAR having an amino acid sequence, which has been amended by a substitution, a deletion, a truncation and/or an addition, 2) chemically modified uPAR and 3) synthetic molecules comprising the amino acid sequence of uPAR corresponding to the uPA binding site of uPAR. Such substances will occupy the cell

receptor-binding site of uPA and hence prevent uPA from binding to the cell-bound receptor.

2b. Substances interacting with the gp120 binding site of uPA

In a preferred embodiment of the invention, the active substance is a monoclonal or polyclonal antibody to the gp120 binding site of uPA.

3. Substances interacting with gp120

In a preferred embodiment of the invention, the active substance is a monoclonal or polyclonal antibody to gp120, e.g. an antibody to the uPA binding site of gp120.

Formulation of the therapeutic composition

The administration of the composition according to the invention may be performed by any prior art administration method, which is suitable for administering the active substance in question, e.g. a protein, peptide or antibody. Typical administration methods include parenteral, oral, nasal, topical and rectal administration.

The parenteral administration route includes intravenously, intramuscularly and subcutaneously administration, and the method is selected dependent on where the active substance is to be released.

It is to be understood that the composition of the invention may further comprise adjuvants, excipients or other additives or ingredients suitable for such formulations. Such adjuvants, excipients and other additives or ingredients are well-known to the person skilled in the art, and include i.a. solvents, emulsifiers, wetting agents, plastisizers, colouring substances, fillers, preservatives, viscosity adjusting agents, buffering agents, mucoadhesive substances, and the like. Examples of formulation strategies are well-known to the person skilled in the art.

The suitable amount of the biologically interactive substance to be administered depends on the substance in question, on the condition of the subject to be treated,

and further on the age, weight and state of the subject in question. Finding the optimal dose will merely be a matter of routine experimentation to the person skilled in the art.

Definitions

In connection with the present invention, "HIV" is defined as Human Immunodeficiency Virus 1 and 2.

In connection with the present invention, "uPA" means urokinase plasminogen activator.

In connection with the present invention, "uPAR" means urokinase plasminogen activator receptor (same as CD87).

In connection with the present invention the term "gp120" means gene product 120, which is a viral envelope protein present in HIV.

Abbreviations

PAI-1: plasminogen activator inhibitor Type 1
PAI-2: plasminogen activator inhibitor Type 2
PMA: phorbol 12-myristate 13-acetate
EGF: epidermal growth factor
PI-PLC: phosphatidylinositide-phospholipase C

EXAMPLES

Example 1

Inhibition of HIV antigen production using a competitive inhibitor against the uPA binding domain of uPAR

Background:

The anti-uPAR monoclonal antibody (R3) is a competitive inhibitor of uPA binding to uPAR through blocking of the uPA binding site of uPAR (8). Thus, it was investigated whether the R3 antibody was able to inhibit viral production, measured as HIV antigen in cell supernatant. The virus used in this experiment is HIV-1 IIIB, an X4 strain. As a negative control for the influence of antibodies in the infection assays, anti-TNP, an isotypic negative control antibody was used.

Materials and methods:

Peripheral blood mononuclear cells (PBMC's) from a healthy donor were separated by Histopaque density gradient centrifugation (Sigma, St. Louis, MO, USA) and frozen at 10 x 10^6 cells/ml in complete medium (RPMI 1640 (Life technologies, Gibco BRL, USA) containing 25 mM HEPES, GlutaMAX-I, 10% FBS (Life technologies, Gibco BRL, USA), 100 U/ml penicillin-streptomycin (Life technologies, Gibco BRL, USA), 0.1 mg/ml gentamicin (Life technologies, Gibco BRL, USA) and 10 units/ml hIL-2 (Roche)) containing 10% DMSO.

PBMC's were thawed quickly at 37°C and grown for four hours at 37°C. Culture medium was subsequently changed to complete medium at a concentration of 2 x 10^6 cells/ml and cells were stimulated with 5 ug/ml PHA for 72 hours.

PBMC's were centrifuged and resuspended in complete medium at a cell concentration of 2 x 10^6 cells/ml. 400 ul cell suspension were transferred to 7 ml culture tubes and the specific reagent of interest added (see table 1). After one hour of incubation at 37°C, 5% CO_2, 100 x ID_{50} HIV-1 IIIB was added to each tube and the cell mixture incubated at 37°C, 5%CO_2, overnight.

PBMC's were washed three times in RPMI 1640 and resuspended in 2 ml of complete medium containing the specific reagent of interest. 200 ul cell suspension was transferred in octaplicates to a 96-well polystyrene microtiter plate (Nunc-immuno plate, Maxisorp, Nunc, Denmark), thus having a final cell concentration of 80,000 cells per well.

The PBMC's were cultured for 6 days at 37°C in 5% CO_2, and from each well half the supernatant was extracted and freezed for later HIV antigen quantification, and

replaced by fresh medium after 4 and 6 days. HIV antigen output was measured by enzyme-linked immunosorbent assay (ELISA) of 10 ul of the supernatants. In brief, the supernatants were examined for HIV antigen by using a double-antibody sandwich ELISA as described previously (9). Each Maxisorp polystyrene microtiter plate included two dilution series of a standard HIV antigen preparation, and absorbances (490 nm) were expressed relative to this standard preparation (arbitrary units).

Results:

The results are shown in Figure 1. In high concentrations, an increased amount of HIV antigen production was observed. In low concentrations (20 and 2 ng R3/ml), a significant reduction in HIV antigen production was observed.

Discussion:

In this experiment, an inhibitory effect of monoclonal antibodies against uPAR on the synthesis of HIV proteins released to the cell medium was observed. The inhibitory effect was not due to the mere presence of antibodies, as shown in the control anti-TNP experiment, and must therefore be due to the specific interaction of the R3 antibody with the uPA receptor. It is interesting that the strongest inhibitory effect was observed with low concentrations of R3 antibody.

Tube	Concentration of reagent
PLATE 1.	
1.1	No added reagent
1.2	R3, 20 µg/ml
1.3	R3, 2 µg/ml
1.4	R3, 200 ng/ml
1.5	R3, 20 ng/ml
1.6	R3, 2 ng/ml
1.7	No added reagent
1.8	Anti-TNP mAb, 20 µg/ml

Table 1. Final concentration of specific agent

Example 2

Inhibition of HIV using a monoclonal antibody (R5) against the uPA binding domain of uPAR

Background:

The anti-uPAR monoclonal antibody (R5) is capable of binding the preformed uPAR:uPA complex, forming a transient trimolecular assembly, and promoting the dissociation of the uPA/uPAR complex (8). Thus, it was investigated whether the R5 antibody was able to inhibit viral production, measured as HIV antigen in supernatant. The virus used in this experiment is HIV-1 IIIB, an X4 strain. As a negative control for the influence of antibodies in the infection assays, anti-TNP, an isotypic negative control antibody was used.

Materials and methods:

Peripheral blood mononuclear cells (PBMC's) from a healthy donor were separated by Histopaque density gradient centrifugation (Sigma, St. Louis, MO, USA) and frozen at 10 x 10^6 cells/ml in complete medium (RPMI 1640 (Life technologies, Gibco BRL, USA) containing 25 mM HEPES, GlutaMAX-I, 10% FBS (Life technologies, Gibco BRL, USA), 100 U/ml penicillin-streptomycin (Life technologies, Gibco BRL, USA), 0.1 mg/ml gentamicin (Life technologies, Gibco BRL, USA) and 10 units/ml hIL-2 (Roche)) containing 10% DMSO.

PBMC's were thawed quickly at 37°C and grown for four hours at 37°C. Culture medium was subsequently changed to complete medium at a concentration of 2 x 10^6 cells/ml and cells were stimulated with 5 ug/ml PHA for 72 hours.

PBMC's were centrifuged and resuspended in complete medium at a cell concentration of 2 x 10^6 cells/ml. 400 ul cell suspension were transferred to 7 ml culture tubes and the specific reagent of interest added (see table 2). After one hour of incubation at 37°C, 5% CO_2, 100 x ID_{50} HIV-1 IIIB was added to each tube and the cell mixture incubated at 37°C, 5%CO_2, overnight.

PBMC's were washed three times in RPMI 1640 and resuspended in 2 ml of complete medium containing the specific reagent of interest. 200 ul cell suspension was transferred in octaplicates to a 96-well polystyrene microtiter plate (Nunc-immuno plate, Maxisorp, Nunc, Denmark), thus having a final cell concentration of 80,000 cells per well.

The PBMC's were cultured for 6 days at 37°C in 5% CO_2, and from each well half the supernatant was extracted and freezed for later HIV antigen quantification, and replaced by fresh medium after 4 and 6 days. HIV antigen output was measured by enzyme-linked immunosorbent assay (ELISA) of 10 ul of the supernatants. In brief, the supernatants were examined for HIV antigen by using a double-antibody sandwich ELISA as described previously (9). Each Maxisorp polystyrene microtiter plate included two dilution series of a standard HIV antigen preparation, and absorbances (490 nm) were expressed relative to this standard preparation (arbitrary units).

Results:

The results of HIV infection of donor PBMC's after 4 days is shown in Figure 2. Titration of monoclonal antibody R5 showed continuous inhibition of HIV viral proteins, with the strongest inhibitory effect (a 50% inhibition) at 20 ng/ml of R5. At tenfold lower concentration (2 ng/ml), a modest inhibitory effect was observed.

Discussion:

In this study, the R5 monoclonal antibody was used, which promotes the dissociation of the uPA:uPAR complex. After 4 days of infection, a concentration dependent inhibition of R5 on HIV protein production was observed. The strongest inhibitory effect was observed at 20 ng/ml of R5, while no significant reduction was observed at the highest concentration of R5. The fact that there is a concentration dependent inhibitory effect of antibodies, suggests that uPAR may be involved in more than one aspect of HIV lifecycle. The bimodal effect of R5 indicates that the uPA receptor may be implicated at various stages of the HIV lifecycle, particularly HIV entry and HIV budding from lipid rafts, as these are cell surface events.

PLATE 2	
2.1	No added reagent
2.2	R5, 20 µg/ml
2.3	R5, 2 µg/ml
2.4	R5, 200 ng/ml
2.5	R5, 20 ng/ml
2.6	R5, 2 ng/ml
2.7	Anti-TNP mAb, 20 µg/ml

Table 2. Final concentration of specific agent

Example 3

Potent inhibition of HIV by blocking of multiple sites on uPAR

Background:

More than one uPAR domain is involved in high affinity binding of uPA (10). The effect of a polyclonal uPAR antibody that blocks all sites of interaction between uPA and uPAR in an HIV infection experiment was therefore studied.

Materials and methods:

Peripheral blood mononuclear cells (PBMC's) from a healthy donor were separated by Histopaque density gradient centrifugation (Sigma, St. Louis, MO, USA) and frozen at 10 x 10^6 cells/ml in complete medium (RPMI 1640 (Life technologies, Gibco BRL, USA) containing 25 mM HEPES, GlutaMAX-I, 10% FBS (Life technologies, Gibco BRL, USA), 100 U/ml penicillin-streptomycin (Life technologies, Gibco BRL, USA), 0.1 mg/ml gentamicin (Life technologies, Gibco BRL, USA) and 10 units/ml hIL-2 (Roche)) containing 10% DMSO.

PBMC's were thawed quickly at 37°C and grown for four hours at 37°C. Culture medium was subsequently changed to complete medium at a concentration of 2 x 10^6 cells/ml and cells were stimulated with 5 ug/ml PHA for 72 hours.

PBMC's were centrifuged and resuspended in complete medium at a cell concentration of 2 x 10^6 cells/ml. 400 ul cell suspension were transferred to 7 ml culture tubes and the specific reagent of interest added (see table 3). After one hour of incubation at 37°C, 5% CO_2, 100 x ID_{50} HIV-1 IIIB was added to each tube and the cell mixture incubated at 37°C, 5%CO_2, overnight.

PBMC's were washed three times in RPMI 1640 and resuspended in 2 ml of complete medium containing the specific reagent of interest. 200 ul cell suspension was transferred in octaplicates to a 96-well polystyrene microtiter plate (Nunc-immuno plate, Maxisorp, Nunc, Denmark), thus having a final cell concentration of 80,000 cells per well.

The PBMC's were cultured for 6 days at 37°C in 5% CO_2, and from each well half the supernatant was extracted and freezed for later HIV antigen quantification, and replaced by fresh medium after 4 and 6 days. HIV antigen output was measured by enzyme-linked immunosorbent assay (ELISA) of 10 ul of the supernatants. In brief, the supernatants were examined for HIV antigen by using a double-antibody sandwich ELISA as described previously (9). Each Maxisorp polystyrene microtiter plate included two dilution series of a standard HIV antigen preparation, and absorbances (490 nm) were expressed relative to this standard preparation (arbitrary units).

Results:

These HIV infection experiments show a very strong inhibitory effect of polyclonal uPAR antibodies on HIV protein production in PBMC's. In particular, at a concentration of 2 ug/ml, a 98% reduction in HIV proteins in supernatant was observed. The inhibitory effect of uPAR antibodies was dependent on the concentration, indicating a therapeutic window for the inhibition of HIV (Figure 3).

Discussion:

It was investigated whether a polyclonal uPAR antibody affects the production of HIV proteins in an infection assay. After four days of incubation with 2 ug/ml anti-uPAR antibody, it was not possible to detect the presence of HIV. At other concentrations (Figure 3), between 40 and 80 percent inhibition was observed. The

strong effect of the polyclonal antibody may indicate a synergistic effect of using multiple antibodies binding sites on uPAR. Furthermore, as uPAR is known to interact with other molecules and receptors (e.g. (11), CR3 (Mac-1, CD11b/CD18)(12) and other integrins (13)), and the interaction of uPAR with other receptors may influence the role of uPAR in HIV infection. These results strongly indicate that the uPA receptor is involved in HIV pathogenesis, and that antibodies' blocking the uPAR receptor is a target for intervening against HIV infection and possible could be used to stop or decrease disease progression in HIV infected individuals.

	Final concentrations
3.1	No added reagent
3.2	pAb, 20 µg/ml
3.3	pAb, 2 µg/ml
3.4	pAb, 200 ng/ml
3.5	pAb, 20 ng/ml
3.6	pAb, 2 ng/ml
3.7	No added reagent
3.8	Anti-TNP mAb, 20 µg/ml

Table 3. Final concentration of specific agent

Example 4

Effect of uPA on HIV replication in U937 cells.

Background:

To address the question whether uPA influences HIV infection, an infection experiment using the pro-monocyte cell line u937 was set up.

Materials and methods:

The promonocyte cell line U937 were cultured in complete medium (RPMI 1640 (Life technologies, Gibco BRL, USA)) containing 25 mM HEPES, GlutaMAX-I, 10% FBS (Life technologies, Gibco BRL, USA), 100 U/ml penicillin-streptomycin

(Life technologies, Gibco BRL, USA) and 0.1 mg/ml gentamicin (Life technologies, Gibco BRL, USA) at 37°C, 5%CO_2 at a cell concentration of 1 x 10^6 cells/ml.

400 ul of the U937 cell suspensions were transferred to 7 ml culture tubes, and different concentrations of urokinase-type plasminogen activator (uPA) (Immuno Denmark, Denmark) were then added. Western blot analysis of the uPA stock showed the existence of high molecular weight form uPA (HMW-uPA), low molecular weight form uPA (LMW-uPA) and the amino-terminal fragment (ATF) of uPA.

After one hour of incubation at 37°C, 5% CO_2, 100 x ID_{50} HIV-1 IIIB was added to each tube and the cell mixture incubated at 37°C, 5% CO_2, overnight.

Cells were washed three times in RPMI 1640 and resuspended in 2 ml of complete medium containing uPA at different concentrations.

200 ul cell suspension was transferred in octaplicates to a 96-well polystyrene microtiter plate (Nunc-immuno plate, Maxisorp, Nunc, Denmark), thus having a final cell concentration of 40,000 cells per well.

The U937 cells were cultured for 12 days at 37°C in 5% CO_2. Every 3-4 days, 100 ul supernatant was collected; half of the cells were discarded and 150 ul fresh medium was added. HIV antigen output was measured by enzyme-linked immunosorbent assay (ELISA) of 10 ul of the supernatants. In brief, the supernatants were examined for HIV antigen by using a double-antibody sandwich ELISA as described previously (9). Each Maxisorp polystyrene microtiter plate included two dilution series of a standard HIV antigen preparation, and absorbances (490 nm) were expressed relative to this standard preparation (arbitrary units).

Experiments were carried out with both pre- and post-incubation of uPA, relative to the addition of HIV.

Results:

These experiments show that uPA modulates HIV protein production released to the cell medium, and the effect on HIV protein production is correlated to the concentration of uPA. At 1 ug/ml uPA, a 48% increase in HIV protein productivity was observed, while a 56% reduction in HIV protein productivity was observed at 100 ng uPA/ml and a 59% reduction at 10 ng/ml. There was no significant difference between preincubation (Figure 4) and post-incubation with uPA (data not shown).

Discussion:

These experiments show that uPA has both an inhibitory and a stimulatory effect on HIV protein productivity in the pro-monocytic cell line U937, dependent on the concentration used. These data are therefore an indicator of the involvement of uPAR and uPA in HIV infection. One of the possible explanations for this dual observation may be the up regulation of uPAR caused by uPA (14). However, uPA is unlikely to become a drug for treatment of HIV infected even though low concentrations has an inhibitory effect, as high concentrations is stimulating HIV production which may occur locally in the body.

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CLAIMS

1. A therapeutic composition for treating or preventing HIV infection comprising an active substance, which is capable of interacting with a complex of cell-uPAR-uPA-gp120-HIV so as to prevent or reduce the internalisation of HIV into the cell.

2. A composition according to claim 1, wherein the active substance is capable of interacting with the formation of the complex of cell-uPAR-uPA-gp120-HIV.

3. A composition according to claim 1 or 2, wherein the active substance is capable of breaking up at least one of the bonds in the complex of cell-uPAR-uPA-gp120-HIV.

4. A composition according to any of claims 1-3, wherein the active substance is capable of bonding or adhering to the complex of cell-uPAR-uPA-gp120-HIV.

5. A composition according to any of claims 1-4, wherein the active substance is selected from the group consisting of substances interacting with uPAR.

6. A composition according to any of claims 1-5, wherein the active substance is selected from the group consisting of substances interacting with uPA.

7. A composition according to any of claims 1-6, wherein the active substance is selected from the group consisting of substances interacting with gp120.

8. Use of a substance for preparing a therapeutic composition for treating or preventing HIV infection, wherein the substance is capable of interacting with the complex of cell-uPAR-uPA-gp120-HIV so as to prevent or reduce the internalisation of HIV.

9. A method of preventing or treating HIV infection comprising administering to a subject a composition comprising a therapeutically effective dose of an active substance, which is capable of interacting with the complex of cell-uPAR-uPA-

gp120-HIV so as to prevent or reduce the internalisation of HIV.

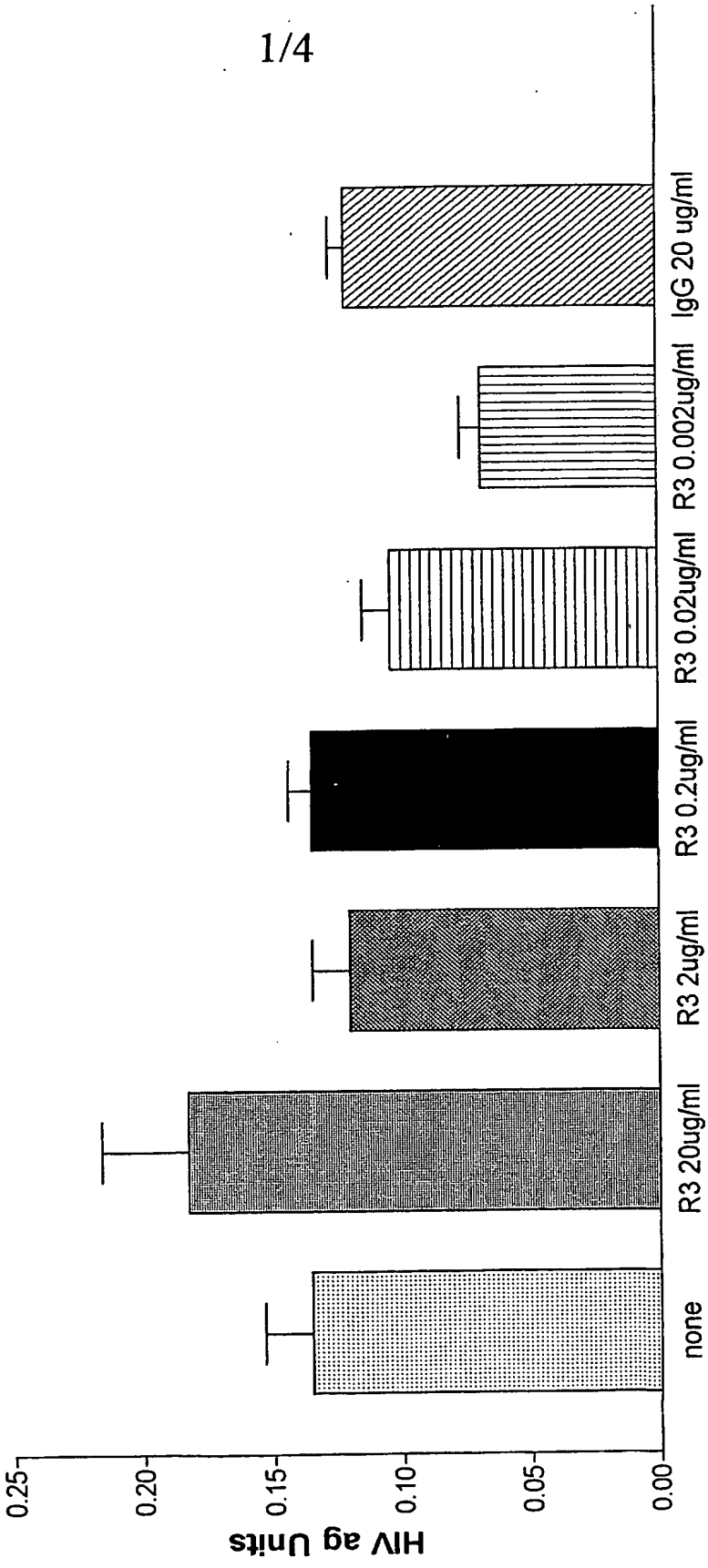
anti R3mab effect on HIV ag production on day 4
HIV ag Units
0.25
0.20
0.15
0.10
0.05
0.00
none
R3 20ug/ml
R3 2ug/ml
R3 0.2ug/ml
R3 0.02ug/ml
R3 0.002ug/ml
IgG 20 ug/ml

Figure 1

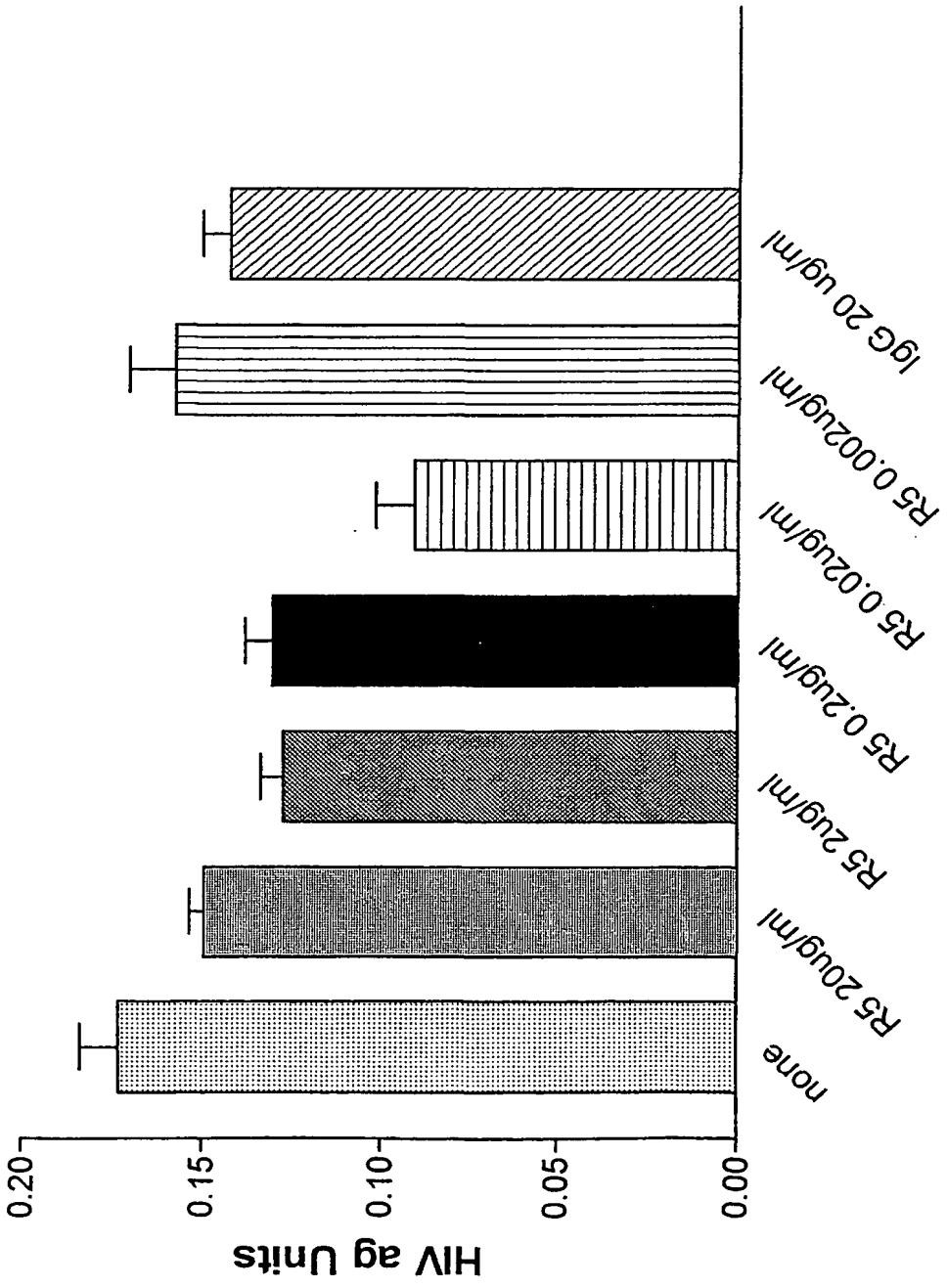
anti R5 mAb effect on HIV ag production on day 4
HIV ag Units
0.00
0.05
0.10
0.15
0.20
none
R5 20ug/ml
R5 2ug/ml
R5 0.2ug/ml
R5 0.02ug/ml
R5 0.002ug/ml
IgG 20 ug/ml

Figure 2

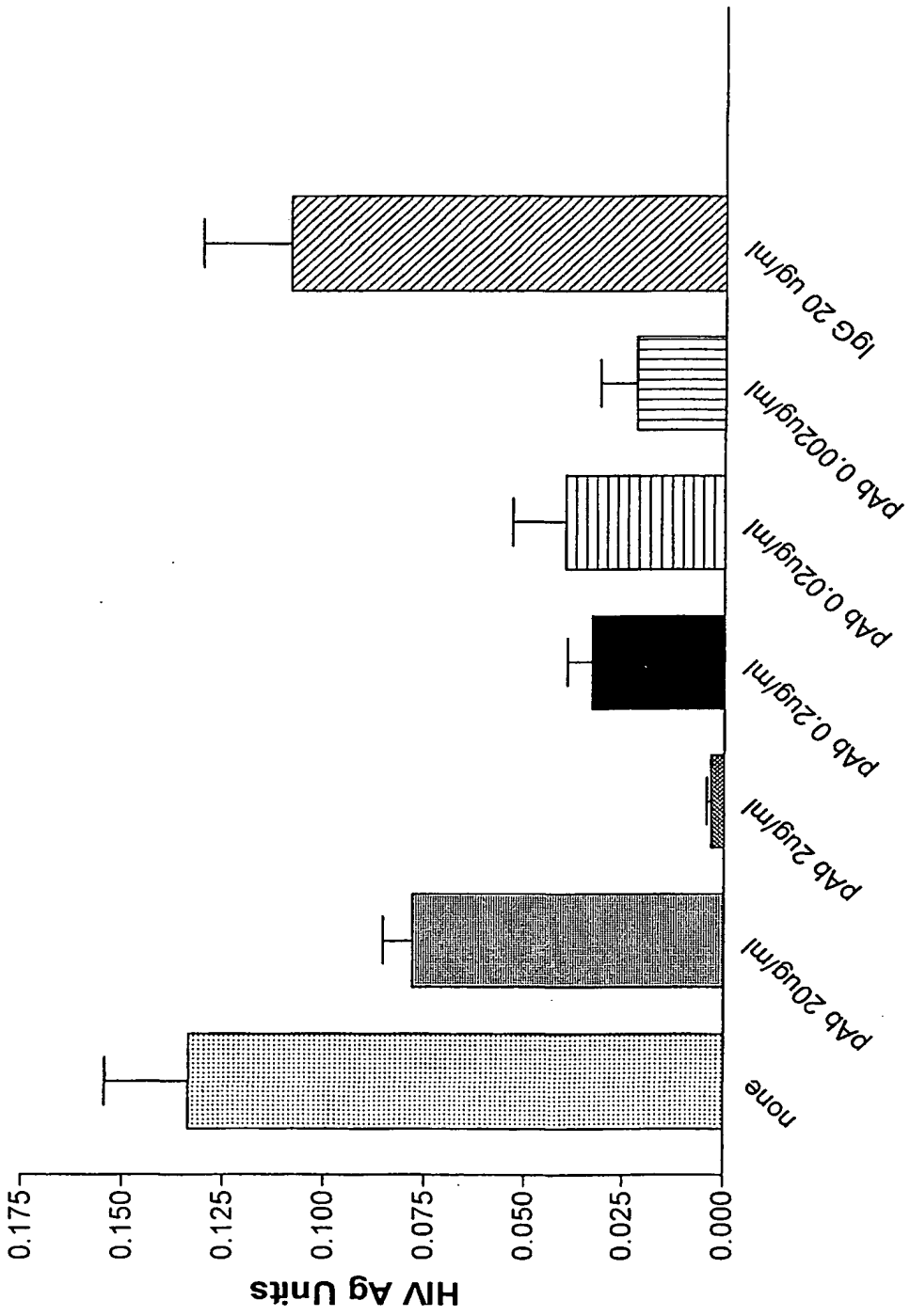
anti CD87 pAb effect on HIV ag production on day 4
HIV Ag Units
0.175
0.150
0.125
0.100
0.075
0.050
0.025
0.000
none
pAb 20ug/ml
pAb 2ug/ml
pAb 0.2ug/ml
pAb 0.02ug/ml
pAb 0.002ug/ml
IgG 20 ug/ml

Figure 3

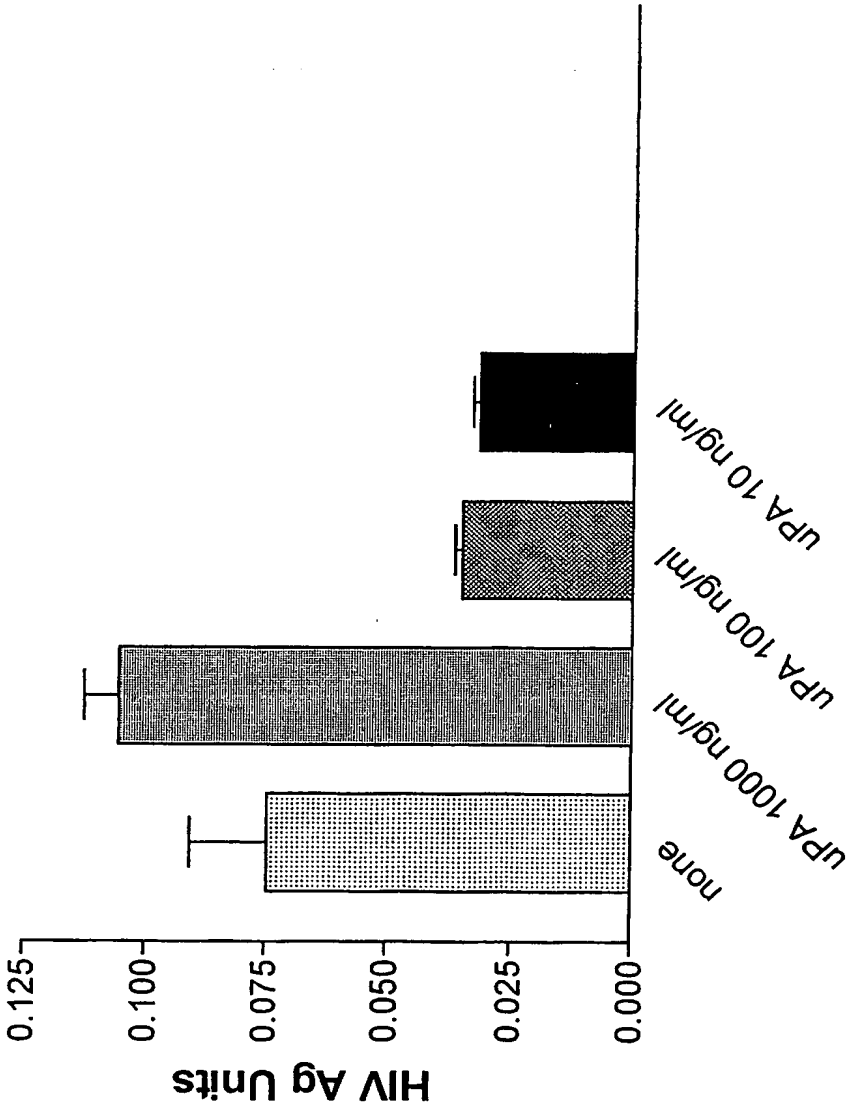
HIV Ag Units
0.125
0.100
0.075
0.050
0.025
0.000
none
UPA 1000 ng/ml
UPA 100 ng/ml
UPA 10 ng/ml

Figure 4

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK 01/00525

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/395 A61K38/17 A61K39/42 A61P31/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal, WPI Data, PAJ, MEDLINE, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HANDLEY MARK A ET AL: "A role of urokinase-type plasminogen activator in human immunodeficiency virus type 1 infection of macrophages." JOURNAL OF VIROLOGY, vol. 70, no. 7, 1996, pages 4451-4456, XP002166382 ISSN: 0022-538X cited in the application the whole document	1-9
X	WO 96 13160 A (NEW ENGLAND DEACONESS HOSPITAL) 9 May 1996 (1996-05-09) page 1, line 24 -page 6, line 25; claims 1-21	1-9

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 November 2001	05/12/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Le Flao, K

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BNSDOCID <WO 0209753A1 I >

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK 01/00525

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	HANDLEY M A ET AL: "Urokinase (uPA) cleaves gp120 of HIV-1 and promotes viral infectivity." CLINICAL RESEARCH, vol. 42, no. 2, 1994, page 155A XP000993243 Meeting of the American Federation for Clinical Research;Baltimore, Maryland, USA; April 29-May 2, 1994 ISSN: 0009-9279 abstract	1-9
A	MURALI RAMA ET AL: "Altered levels of urokinase on monocytes and in serum of children with AIDS: Effects on lymphocyte activation and surface marker expression." JOURNAL OF LEUKOCYTE BIOLOGY, vol. 64, no. 2, August 1998 (1998-08), pages 198-202, XP000993187 ISSN: 0741-5400 the whole document	1-9
P,A	WO 01 38871 A (EUGEN OLSEN JESPER) 31 May 2001 (2001-05-31) claims 1-11	1-9
P,X	WADA MANABU ET AL: "Amino-terminal fragment of urokinase-type plasminogen activator inhibits HIV-1 replication." BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 284, no. 2, 8 June 2001 (2001-06-08), pages 346-351, XP002183644 ISSN: 0006-291X the whole document	1-9

BNSDOCID: <WO 0209753A1 I >

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-9

Present claims 1-9 relate to an extremely large number of possible compounds. In fact, the claims contain so many possible compounds that a lack of conciseness within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible. Consequently, the search has been carried out for those parts of the application which do appear to be concise, namely antibodies anti gp120, anti uPA, anti uPAR and uPA inhibitors such as PAI-1 have been searched.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int[redacted]tional Application No
PCT/DK 01/00525

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9613160 A	09-05-1996	WO 9613160 A1	09-05-1996
WO 0138871 A	31-05-2001	AU 1693301 A	04-06-2001
		WO 0138871 A1	31-05-2001

BNSDOCID <WO 0209753A1 I >